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Proteins, Detection of

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Introduction

After polyacrylamide gel electrophoresis, it is essential that separated protein zones be detected for subsequent analysis, whether this is to be done by simple visual inspection or by quantitative computerized densitometry. In the early days of electrophoresis, methods for the detection of separated zones (ultraviolet absorption, Schlieren optics) were limited and insensitive. The subsequent development of organic dyes able to react with proteins made stains such as Bromophenol Blue and Amido Black 10B popular. In particular, Coomassie Brilliant Blue was for many years the method of choice for protein detection following gel electrophoresis owing to its relatively high sensitivity. However, the need for increased sensitivity resulted in the development of a group of staining methods based on the use of silver (approximately 0.1 ng of protein per band). Recently, there has been a renewed interest in the use of fluorescent methods of protein detection as they provide high sensitivity equivalent to silver staining combined with excellent linearity and extended dynamic range. Detection methods based on the use of radiolabelling also provide high sensitivity but cannot be applied in all situations. Finally, methods are available for the detection of groups of proteins with specific post-translational modifications, for example glycoproteins, phosphoproteins and lipoproteins.

Fixation

After electrophoresis is complete, the gel is removed from the apparatus for localization of the separated zones. Procedures have been described for the direct visualization of unfixed proteins within gels. However, for the majority of protein detection methods it is necessary to precipitate and immobilize (i.e. 'fix') the separated proteins within the gel and to remove any nonprotein components which might interfere with subsequent staining. Gels that are to be used for visualization of enzymatic activity of the separated proteins must not be fixed. The best general purpose fixative is 20% w/v trichloroacetic acid (TCA) as it gives effective precipitation of most proteins. Acid methanol (or ethanol), typically a solution containing 10% v/v acetic acid, 45% v/v methanol, and 45% deionized water, is often used for gel fixation, but it should be noted that this can be ineffective for small proteins, basic proteins and glycoproteins. Aqueous solutions of reagents such as 5% w/v formaldehyde or 2% w/v glutaraldehyde can be used to cross-link proteins covalently to the gel matrix, but this is not a commonly used approach.

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Coomassie Brilliant Blue

The most popular general protein-staining procedures following gel electrophoresis are based on the use of the non-polar, sulfated triphenylmethane Coomassie stains, developed for the textile industry, Coomassie Brilliant Blue (CBB) R-250 is most often used and requires an acidic medium for electrostatic interaction between the dye molecules and the amino groups of proteins. Staining is usually carried out using 0.1% w/v CBB R-250 in the same acid methanol solution used for fixation (10% acetic acid, 45% methanol). Depending on gel thickness and polyacrylamide concentration, staining can take from 30 min to several hours. In practice, it is often convenient to stain the gel overnight and then destain it by several changes in the same acid methanol solution until intense blue protein zones can be seen against a clear background. This method is able to detect a minimum of around 100 ng protein per band (**Figure 1**), so that for complex mixtures containing several hundred components, it is necessary to load relatively high amounts of total protein ($> 50\ \mu g$).

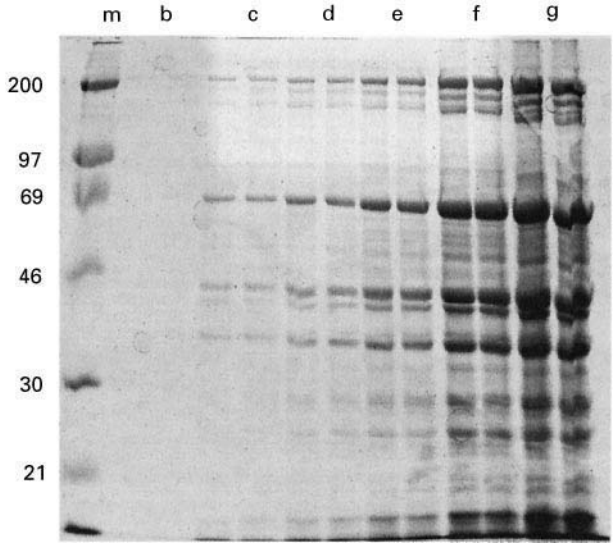

Figure 1 SDS–PAGE separation of human heart proteins (lanes b–g). Lane (a) contains the molecular weight marker proteins and the scale at the left indicates protein size in kDa. The gel has been stained with Coomassie Brilliant Blue R-250. The sample protein loadings were (b) 1 μg, (c) 5 μg, (d) 10 μg, (e) 25 μg, (f) 50 μg, (g) 100 μg.

More sensitive staining (down to 10 ng protein per band) can be achieved using the dimethylated form of the dye, CBB G-250, as a 0.1% w/v colloidal dispersion in 2% w/v phosphoric acid, 10% w/v ammonium sulfate, and 20% v/v methanol. An additional advantage of this method is that the colloidal dye only binds to the separated proteins as it is unable to penetrate the gel matrix. This means that no destaining step is required and the intensity of staining can be controlled by visual inspection during the staining process. Related dyes such as Acid Violet 17, Serva Violet 49 and Fast Green FCF also form colloids in strongly acidic solutions and stain proteins in gels with low background.

Silver Staining

Silver has been known to be able to develop images for over two hundred years, first being usefully exploited in photography and then rapidly adopted for use in histological staining procedures. The ability of silver to detect proteins following their separation by gel electrophoresis was first recognized by Merril and his colleagues in 1979. Subsequently, more than a hundred silver-staining procedures have been described and this group of methods has become the standard approach for the sensitive detection of gel-separated proteins. However, certain classes of proteins, such as calcium-binding proteins and glycoproteins, stain rather poorly, with an inverse relationship between the intensity of silver staining and the proportion of the molecule that is composed of carbohydrate. Pre-staining with cationic dyes prior to silver staining can significantly improve the sensitivity of detection of glycoproteins.

Depending on the method, silver staining is between ten and a hundred times more sensitive than staining with CBB R-250, and is able to detect low nanogram amounts of protein. There can be problems in using silver staining as a quantitative procedure as it is known to be non-stoichiometric. However, staining intensity is linear over a 40-fold range, comparing well with the 20-fold linear range of CBB R-250. Above this limit, the stain becomes non-linear, resulting in saturation and even negative staining of bands and spots at very high protein concentrations, making quantitation of such protein zones impossible. In a two-dimensional electrophoresis study of human leukocyte proteins, over 200 spots were observed to have coefficients of variation less than or equal to 15% when data from replicate patterns were analysed. In dilution experiments, the majority ($> 80\%$) of the proteins were found to have a linear relationship between the amount of protein loaded and the spot volume. An additional problem with the quantitation of silver staining is that the relationship between staining intensity and protein concentration may be different for each protein. However, it is often forgotten that this is also the case for staining with CBB R-250.

All silver-staining procedures depend on the reduction of ionic silver to its metallic form, but the precise mechanism involved in the staining of proteins has not been fully established. It has been proposed that silver cations complex with protein amino groups, particularly the ε-amino group of lysine, and with sulfur residues of cysteine and methionine. However, staining cannot be attributed exclusively to specific amino groups suggesting that some other component of protein structure is also responsible for differential protein staining.

Procedures for silver staining can be grouped into two main types depending on the chemical state of the silver when used for impregnating the gel. The first group comprises alkaline methods based on the use of ammoniacal silver or diamine solution, prepared by adding silver nitrate to sodium-ammonium hydroxide mixture. Copper can be included in these diamine methods to give increased sensitivity, probably by a mechanism similar to the Biuret reaction. The silver ions complexed to proteins in the gel are then developed by reduction to metallic silver with formaldehyde in an acidified environment, usually using citric acid. In the alternative group of methods, silver nitrate in a weakly acidic (around pH 6.0)

solution is used for gel impregnation. Development is subsequently achieved by the selective reduction of ionic silver to metallic silver by formaldehyde made alkaline with either sodium carbonate or sodium hydroxide. Any free silver is washed out of the gel prior to development to prevent precipitation of silver oxide that would result in high background staining.

The majority of silver staining procedures are monochromatic, resulting in dark brown to black protein zones. However, if the development time is extended with saturation of the zones of highest protein concentration, then colour effects can be produced. In a comparative study of several methods based on both the silver diamine and silver nitrate approaches, the most rapid procedures were found to be generally less sensitive than those which were more time-consuming. The use of glutaraldehyde pre-treatment of the gel and silver diamine as the silvering agent were found to be the most sensitive and example of a gel stained with a method of this type is shown in **Figure 2**.

Increasingly, proteins are being visualized in gels for subsequent identification and characterization by techniques such as mass spectrometry. In this case, glutaraldehyde cannot be used and silver-staining protocols that omit this reagent must be used. However, this modification results in a decrease in sensitivity and uniformity of staining as well as an increase in background.

It is a common experience that silver-staining procedures can give rise to problems when based on the use of laboratory-prepared reagents. If care is not taken with the use of high-purity water, reagents and glassware, then problems of high background staining, surface 'mirror' effects and poor reproducibility can be experienced. Many of these problems can be alleviated using one of the commercially available silver-staining kits (for example from Amersham-Pharmacia Biotech, Bio-Rad Laboratories, Richmond, CA, USA).

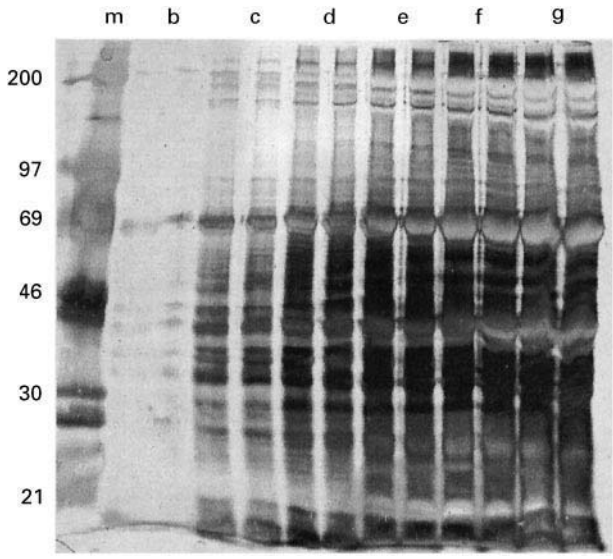

Figure 2 SDS–PAGE separation of human heart proteins (lanes b–g). Lane (a) contains the molecular weight marker proteins and the scale at the left indicates protein size in kDa. The gel has been silver stained. The sample protein loadings were (b) 1 μg, (c) 5 μg, (d) 10 μg, (e) 25 μg, (f) 50 μg, (g) 100 μg.

Reverse Stains

One disadvantage of the standard protocols for staining with Coomassie Blue dyes and silver is that it is essential to use a fixation step prior to staining. Unfortunately, this can result in reduced recovery of proteins from the gel for subsequent chemical characterization. Reverse stains have been developed to specifically overcome this problem. The result of these stains is a semi-opaque background on the gel surface, while the proteins are visible as transparent zones using back-lighting. The process of staining is rapid, requiring generally between 5 and 15 min. After staining, the proteins can be eluted after chelation of the metal ions with agents such as EDTA. It should be noted that reverse stains are not suitable for quantitative applications.

A variety of reverse-stain methods suitable for visualizing proteins after SDS–PAGE have been described. The most popular methods have been those using potassium chloride, copper chloride and zinc chloride, with the last being the most sensitive. The zinc imidazole-staining method is quite sensitive, with a limit of detection of around 10 ng protein per band. In the presence of imidazole, free or weakly bound zinc ions are readily precipitated as zinc imidazole, while tightly bound ions associated with proteins do not precipitate. This results in clear protein zones on an opaque background.

Fluorescent Detection Methods

Many of the problems inherent in the quantification of gel-separated proteins visualized by silver staining can be overcome using detection methods based on the use of fluorescent compounds. This group of methods is highly sensitive and generally exhibits excellent linearity and a high dynamic range, making it possible to achieve good quantitative analysis, particularly if a suitable imaging device is used.

Two approaches can be used, the first being to couple the proteins with a fluorescent-labelled compound prior to electrophoresis. Examples of such compounds are: dansyl chloride; fluorescamine (4-phenyl-[furan-2(3H)-1-phthalan]-3,3′-dione);

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o-phthaldialdehyde (OPA) + a thiol; and MDPF (2-methoxy-2,4-diphenyl-3(2H)-furanone). The last reagent has a reported sensitivity of 1 ng protein per band and is linear over the range 1–500 ng protein per band.

The main disadvantage of pre-electrophoretic staining procedures is that they can cause protein-charge modifications, for example fluorescamine converts an amino group to a carboxyl group when it reacts with proteins. Such modifications usually do not compromise SDS–PAGE unless a large number of additional charged groups are introduced into the protein. However, they result in altered mobility during other forms of electrophoresis, giving rise to altered separations by native PAGE, IEF and two-dimensional electrophoresis. Recently, compounds that react with cysteine or lysine residues have been described and used successfully for two-dimensional electrophoresis separations. The cysteine-reactive reagent monobromobimane has been used to label proteins prior to analysis by two-dimensional electrophoresis. Using a cooled CCD camera to measure fluorescence, the limit of detection was found to be 1 pg protein per spot.

In an alternative approach, two amine-reactive dyes (propyl Cy3 and methyl Cy5) have been synthesized and used to label *Escherichia coli* proteins prior to electrophoresis. These cyanine dyes have an inherent positive charge, which preserves the overall charge of the proteins after dye coupling. The two dyes have sufficiently different fluorescence spectra that they can be distinguished when they are present together. This allowed two different protein samples, each labelled with one of the dyes, to be mixed together and subjected to two-dimensional electrophoresis on the same gel. This method, which has been termed 'difference gel electrophoresis (DIGE)', has great potential for improving the efficiency of detection of differences in two-dimensional electrophoresis protein profiles between different samples.

For two-dimensional electrophoresis, one approach to overcoming the problems associated with charge modification during the IEF dimension is to label the proteins while present in the first dimension gel after IEF, prior to the second dimension separation by SDS–PAGE. Two fluorescent labels that have been used in this way are MDPF and a fluorescent maleimide derivative.

The alternative approach, which also overcomes the problem of protein-charge modifications, is to label the proteins with fluorescent molecules such as 1-aniline-8-naphthalenesulfonate (ANS) and OPA after the electrophoretic separation has been completed. However, these two methods suffer the disadvantage of relative insensitivity. Recently, two post-electrophoretic fluorescence staining reagents, SYPRO orange and red (Molecular Probes, Eugene, Oregon, USA), have been described. These stains have a very high sensitivity (1–2 ng protein per band) and excellent linearity with a high dynamic range. Using a fluorescence imaging device, the SYPRO dyes have been shown to be linear over three orders of magnitude in protein quantity. The other advantage of this method is that staining can be achieved in only 30 min, compared with staining with silver and CBB R-250 which can take from 2 h to overnight. Gels can be stained without fixation so that they can be subjected to subsequent Western blotting procedures. However, staining with these reagents requires that the proteins are complexed with SDS, so that if the gels are fixed prior to staining or electrophoresis is carried out in the absence of this detergent, then the gels must be incubated in a solution of SDS prior to staining. An SDS–PAGE gel separation visualized using SYPRO red is shown in **Figure 3**.

Metal Chelate Stains

This recently developed group of stains have been developed specifically for compatibility with characterization methods such as mass spectrometry as they do not use reagents such as glutaraldehyde or formaldehyde which reduce their efficacy. Although a stain of this type, using the pink bathophenanthroline disulfonate/ferrous complex, was described over twenty

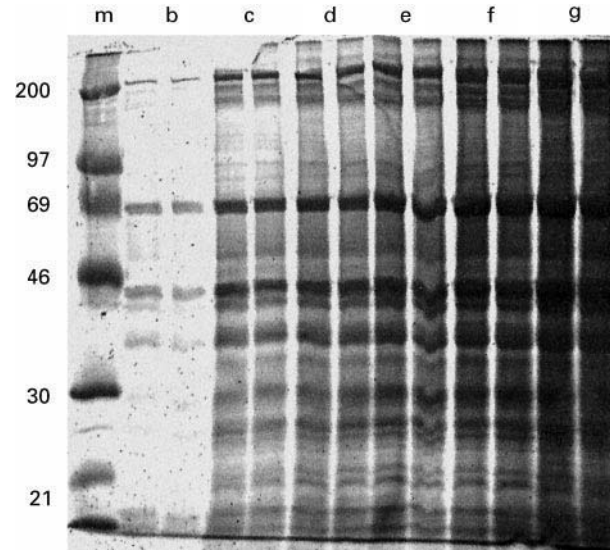

Figure 3 SDS–PAGE separation of human heart proteins (lanes b–g). Lane (a) contains the molecular weight marker proteins and the scale at the left indicates protein size in kDa. The gel has been stained with SYPRO red. The sample protein loadings were (b) 1 μg, (c) 5 μg, (d) 10 μg, (e) 25 μg, (f) 50 μg, (g) 100 μg.

years ago, its insensitivity (600 ng protein) ensured its lack of acceptance. Sensitivity can be increased by introducing ^{59}Fe into the complex, but only to a level equivalent of colloidal Coomassie Blue staining.

The poor performance of these dyes resulted in a recent investigation of luminescent metal chelate stains. Such stains utilizing metal chelates complexed to certain transition metal ions (e.g. europium, terbium, and ruthenium) offer much greater sensitivity compared to the previous colorimetric methods. Of particular interest is SYPRO Ruby, a proprietary ruthenium-based metal chelate stain (Molecular Probes, Eugene, Oregon, USA). This allows one-step, low background staining of proteins in polyacrylamide gels without resorting to lengthy destaining procedures. The linear dynamic range of this dye extends over three orders of magnitude, thus surpassing silver and Coomassie stains in performance. Its sensitivity is claimed to be up to thirty times more sensitive than silver staining. Moreover, staining times (unlike silver protocols) are not critical and staining can be carried out overnight without over-development.

Radioactive Detection Methods

Metabolic labelling of proteins with a radiolabelled amino acid prior to their separation by gel electrophoresis represents a very sensitive method for the detection of proteins and is ideal for the analysis of protein synthetic events occurring in response to an experimental intervention. This approach is most commonly used in combination with *in vitro* cell culture systems, but it is also possible to radiolabel synthetically the proteins of small pieces of fresh tissue in this way. In this method, the cells or tissue are incubated in the presence of the radiolabelled amino acid for a period of time, normally between 3 and 24 h. It is important to use a tissue culture medium that has been depleted of the amino acid used for radiolabelling. The most commonly used amino acids for radiolabelling are [^{35}S]-methionine and [^{14}C]-leucine. [^{3}H]-amino acids can be used, but these are more difficult to detect due to the weak energy of their beta emissions. Methods are also available for synthetic radiolabelling to detect specific post-translational modifications of proteins.

Proteins can also be radiolabelled post-synthetically, prior to their separation by gel electrophoresis, using a variety of methods such as radioiodination with ^{125}I or reductive methylation with [^{3}H]-sodium borohydride. However, most of these methods result in significant charge modification of the target proteins, generally precluding their use for electrophoretic techniques other than SDS–PAGE.

Following electrophoresis of radiolabelled proteins, the gel must normally be dried prior to detection of the radioactive zones. Thin gels cast on plastic supports can be dried, after equilibration in 3% w/v glycerol, in air or in an oven at 40–50°C. It is also possible to air-dry gels which have not been cast on supports. These should be equilibrated in 3% w/v glycerol and placed between two cellophane sheets supported in a plastic frame. The gels are then dried in hot air at 40–50°C; the process usually taking 2 or 3 h. The best method for drying gels which are not on supports is by heating them under vacuum. Gels should be soaked in 3% w/v glycerol prior to drying. Gradient polyacrylamide gels are particularly prone to cracking and these can be protected by soaking in a solution containing 1% w/v glycerol and 2% v/v dimethyl sulfoxide (DMSO). Gels can be dried down onto filter paper or onto cellophane. A temperature of 80°C is normally used, but it is better to use a lower temperature (40–60°C) for gels at risk of cracking (i.e. thick, high percentage or gradient gels).

Radiolabelled proteins are most easily detected by direct autoradiography, in which the dried gel is placed in contact with X-ray film and exposed for the appropriate time. This method works satisfactorily for isotopes such as ^{14}C, ^{35}S, ^{32}P and ^{125}I, but is not suitable for ^{3}H owing to its low-energy beta-emissions which are not able to penetrate the gel matrix. Much sensitive detection can be achieved using flourography in which the gel is impregnated with a scintillant, such that low-energy beta particles excite the fluor molecules to emit photons which can be detected on a suitable (usually blue-sensitive) X-ray film. In the original procedure, 2,5-diphenyloxazole (PPO) which must be dissolved in DMSO, was used. However, fluorography with commercially available enhancers is simpler and less tedious than the original PO–DMSO method, and produces equivalent results. Pre-exposure of the X-ray film to a brief flash of light (approximately 1 ms) increases the sensitivity of fluorography by two- or threefold. The use of an intensifying screen and exposure at low temperature (−70°C) also result in a significant increase in sensitivity.

Techniques of autoradiography and fluorography are simple and require little specialized equipment, apart from the access to darkroom facilities. However, prolonged exposure times are often required to achieve the desired level of sensitivity of protein detection. Moreover, the nonlinear response of X-ray film and its limited dynamic range present severe problems to accurate quantitation. To overcome these problems several devices for detecting radiolabelled proteins directly in gels have been described. The best and most practical of these

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approaches are imaging devices based on the use of photostimulable storage phosphor-imaging screens.

Detection of Specific Biological Compounds

Detection of Glycoproteins

Proteins with limited glycosylation can be detected following gel electrophoresis with the general protein stains such as CBB R-250 and silver. However, such staining gives no direct indication that these proteins are glycosylated and the methods are much less sensitive if the proteins are more highly glycosylated. Proteoglycans are usually stained with cationic dyes, such as Alcian Blue or Toluidine Blue, which bind to the negatively charged glycosaminoglycan side chains. Glycoproteins have generally been detected using variations of the Schiff base reaction, involving oxidation with periodic acid followed by staining with Schiff reagent, Alcian Blue or a hydrazine derivative. A twofold increase in sensitivity can be achieved with methods in which Alcian Blue is used as the primary staining agent followed by subsequent enhancement using a neutral silver-staining protocol.

An alternative approach to the analysis of glycosylated proteins is to radiolabel then *in vitro*, followed by gel electrophoretic separation of the radiolabelled proteins and their detection. *N*-linked sugar labelling can be achieved using [^{3}H]-mannose and terminal *O*-linked *N*-acetylglucosamine can be labelled by galactosyltransferase and UDP-[^{3}H]-galactose.

Probably the most versatile reagents for the characterization of glycosylated proteins following their separation by electrophoresis are radiolabelled, fluorescent or enzyme-conjugated lectins. Although it is possible to use these directly in the gel matrix, much better results are achieved using Western blotting techniques.

Detection of Phosphoproteins

The most commonly used approach to the analysis of protein phosphorylation is to radiolabel cells in culture with either [^{32}P]-orthophosphate or [γ-^{32}P]-ATP. An alternative approach, which avoids the use of radioactive materials, is to use antibodies which are specific to phosphotyrosine, phosphothreonine and phosphoserine in combination with Western immunoblotting.

Detection of Lipoproteins

Lipoproteins can be stained following electrophoresis with Sudan black B. Prenylated proteins can be radiolabelled prior to electrophoresis with [^{3}H]-mevalonolactone, while fatty acylated proteins can be radiolabelled with [^{3}H]-palmitic or [^{3}H]-myristic acid.

Detection of Enzymes

It is generally considered that specific enzyme activities can only be visualized following gel electrophoresis if native conditions have been used. However, there are several reports demonstrating that SDS-denatured proteins can also be visualized provided that it is possible to achieve at least partial renaturation of their spatial configuration. Such renaturation is most effective if disulfide bonds are not essential for enzymic activity and if the native protein is not composed of subunits of different molecular weights. Pre-electrophoresis of gels is usually recommended to remove unreacted acrylamide monomers and catalysts.

Enzyme staining can be achieved by incubating the unfixed gel in a solution of the appropriate reagents using either fluorogenic or chromogenic substrates. This method works well if the final reaction product is insoluble. However, a soluble reaction product will rapidly diffuse resulting in loss of resolution. It is generally preferable to use a print or gel overlay technique. In this approach, the substrates and other reagents are either impregnated into a filter or included in a thin layer of agarose or polyacrylamide gel cast on a glass or plastic support. The overlay is then placed in direct contact with the surface of the separation gel and following a suitable period of incubation, the enzymic activity is visualized on the overlay. Methods are available for the visualization of a large number of enzyme activities following gel electrophoresis.

See also: **III/Proteins:** Capillary Electrophoresis; Electrophoresis. **Electrophoresis:** Detection Techniques: Staining; Autoradiography and Blotting

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Staining

See **II / ELECTROPHORESIS / Detection Techniques: Staining, Autoradiography and Blotting**

Theory of Electrophoresis

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Principles

Electrophoresis is a very separation technique which involves the separation of charged species (molecules) on the basis of their movement under the influence of an applied electric field. It is widely used by chemists and biochemists in studies related to medical technology, environmental research, food and water analysis, pollution control and forensic investigations. The development and applications of electrophoretic separation methods are an example of the fruitfulness of using physical methods in tackling biological and biochemical problems.

The migration of charged colloidal particles in an electric field was originally given the name cataphoresis or electrophoresis. Because there has been some diversity of opinion about the definition of a colloid, and thus about the distinction between colloidal and molecular systems, there has also been some difference of opinion as to how widely the term 'electrophoresis' should be used. Some authors prefer the term ionophoresis to describe the movement of relatively small molecules or ions under such conditions.

The 1940s and 1950s witnessed very rapid developments in the applications of methods making use of the migration of particles in an electric field. These applications covered the whole range of particle sizes from the largest protein molecules to small molecules like amino acids, sugars (at high pH) and even simple inorganic ions, using the sample types of procedures and apparatus. Although it is not a form of chromatography, the differences in the rates of migration of the charged particles provide a powerful means of separating biocolloids such as proteins, polysaccharides and nucleic acids, as well as for the characterization of their components. For these reasons, and also for historical reasons, it is now general practice to use the term 'electrophoresis' to refer to all these procedures. Electrophoresis pertains to the transport of electrically charged particles – ions, colloids, macromolecular ions or particulate matter – in an electric field.